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Pancreatic Islet Transplantation Using Vascularised Chambers Containing Nerve Growth Factor Ameliorates Hyperglycaemia in Diabetic Mice

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Key Words

Type 1 diabetes · Islet transplantation · Vascularised chamber model · Gelatin sponge · Nerve growth factor

Abstract

Intraportal islet transplantation has shown initial promise for the treatment of type 1 diabetes. However, the portal vein site is associated with complications such as thrombosis and hepatic steatosis, leading to transplant failure. The aims of this study were to (1) test the feasibility of an alternative islet transplantation method that utilises a FDA-approved gelatin sponge as a novel islet carrier and (2) assess if exogenous addition of nerve growth factor (NGF) has any additional beneficial effects on graft performance in diabetic mice. Mice were rendered diabetic by a single intraperitoneal injection of streptozotocin. Five hundred syngeneic islets were seeded onto a Gelitaspon® disc in the presence or absence of NGF, and placed into a silicone chamber surrounding the femoral neurovascular pedicle. Islet function was assessed by weekly monitoring of blood glucose levels and an intraperitoneal glucose tolerance test performed at the end of the study. Chambers were harvested for further histological analysis. Four of five mice transplanted with islets seeded onto Gelitaspon with NGF showed a significant reduction in blood glucose levels by 4 weeks after transplantation, and demonstrated a response similar to non-diabetic mice when tested with an intraperitoneal glucose tolerance test. Chamber tissue from this group contained islets with insulin-producing β cells adjacent to the vascular pedicle. Islets seeded onto Gelitaspon with NGF and sited on femoral vessels using a tissue-engineering chamber offer an alternative method for islet transplantation in diabetic mice. This may have potential as a method for clinical islet transplantation.

Abbreviations used in this paper

APES	3-aminopropyltriethoxysilane
bFGF	basic fibroblast growth factor
BG	blood glucose
DAB	diaminobenzaldehyde
HE	haematoxylin and eosin
IPGTT	intraperitoneal glucose tolerance test
NFBG	non-fasting blood glucose
NGF	nerve growth factor
NHMRC	national health and medical research council
PBS	phosphate buffered saline
STZ	streptozotocin
T1D	type 1 diabetes
TH	hydroxylase

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Introduction

Type 1 diabetes (T1D) is an autoimmune-mediated disorder that destroys the insulin-producing β cells of the pancreas [Wild et al., 2004]. At present, treatment for patients with T1D involves rigorous insulin supplementation and blood glucose (BG) monitoring. Although this treatment regimen does improve the condition, patients can still experience hypoglycaemic attacks, and it offers no guarantee that they will avoid secondary complications such as retinopathy, nephropathy, neuropathy and accelerated cardiovascular disease in later life [Shamoon et al., 1993; Genuth et al., 1999; Marzorati et al., 2007]. An improved glycaemic control can be restored in T1D patients with β cell replacement, thereby preventing or delaying the onset of associated secondary complications.

The replacement of β cell mass may be achieved through whole pancreas transplantation or pancreatic islet transplantation. The former procedure is demanding and patients require a lifelong immunosuppressant regimen. It is often associated with concomitant kidney transplantation, and is preferred in diabetic patients with end-stage renal failure [Robertson et al., 2000]. In 2000, islet transplantation via intraportal infusion into the liver (the Edmonton Protocol) was thought to herald a new era of clinical islet transplantation [Shapiro et al., 2000]. Short-term clinical studies initially reported a 1-year insulin-independent rate of >80% [Froud et al., 2005]. However, the long-term outcome has not been as promising as initial expectations [Shapiro et al., 2006]. After 5 years, 85% of the patients had well-controlled HbA1c levels, no episodic hypoglycemia and a significant reduction in the daily insulin requirement. However, only 10% of the patients were free from exogenous insulin [Matas et al., 2005; Shapiro et al., 2006]. Today, clinical islet transplantation is restricted to patients with labile T1D. These patients experience rapid, out-of-proportion BG changes despite intensive insulin management and practice.

The site for clinical islet transplantation is the portal vein of the liver. Immunologically, the liver has been considered an advantageous site requiring less immunosuppression as a solid organ transplant compared to other transplanted organs. This, and the early success of autologous islet transplantation, made the liver the ideal site [Najarian et al., 1979; Bromberg et al., 2007]. However, portal hypertension, the risk of haemorrhage, portal vein thrombosis and gall bladder injury, although rare, are all significant complications [Ryan et al., 2004]. Altered liver function and hepatic steatosis have also been reported [Ryan et al., 2004]. The hepatic transplantation site itself also carries a significant morbidity for islets. This site exposes them to an environment containing toxins and immunosuppressive drugs [Balamurugan et al., 2006], and the microenvironment around the engrafted islets may be hypoxic and prone to local inflammation [Barshes et al., 2005]. Some reports also suggest that allogeneic cells dispersed in the portal vein can generate alloantibodies, some of which may be produced by the liver [Campbell et al., 2007]. Thus, the liver may not be the ideal site for islet transplantation.

Experimental islet transplantation studies have investigated several alternative anatomical sites. These include the peritoneum, the epididymal fat pad, muscle, spleen, the mammary fat pad, the omentum and renal capsule [Balamurugan et al., 2006]. More recently, intravascular transplantation of encapsulated islets has been trialed as a possible treatment for T1D, with promising results [Prochorov et al., 2008]. Our preliminary studies in this field have used a tissue-engineering chamber model based on the superficial epigastric pedicle using bFGF-supplemented Matrigel™ as the carrier matrix for the transplanted islets. Our initial results showed relatively poor vascularisation of tissue. As Matrigel is a basement membrane extract derived from the Engelbreth-Holm-Swarm mouse sarcoma means this method has limited use. Gelitaspon®, a biodegradable FDA-approved gelatin sponge has homology to human collagen and may provide a suitable vehicle for islet transplantation. Islet function and survival is dependent on a number of intrinsic factors. One factor is the neurotrophin nerve growth factor (NGF). NGF has been shown to play a regulatory role in β cell function [Polak et al., 1993], in particular in potentiating insulin secretion [Frodin et al., 1995; Rosenbaum et al., 2001], and has been shown to improve β cell function and prolong survival of transplanted mouse islets in vitro and in vivo when used in a bolus dose [Miao et al., 2005, 2006]. More recently, NGF has been shown to have pro-angiogenic and pro-arteriogenic properties [Emanueli et al., 2002]. All of these studies have used NGF at various concentrations as a bolus dose ranging from 50 to 500 ng/ml. However, a dose between 50 and 100 ng/ml can produce a biological response. Gelitaspon in combination with NGF may be a promising alternative method for human islet transplantation.

The aims of this study were to (1) investigate the feasibility of incorporating the femoral neurovascular bundle into a tissue-engineering chamber as an alternative islet transplantation site, (2) investigate the feasibility of utilising a FDA-approved Gelitaspon sponge as the islet carrier and (3) to assess if the addition of 100 ng/ml NGF has any beneficial effects on islet graft function in streptozotocin (STZ)-induced diabetic mice.

Materials and Methods

Animals

Male C57BL/6J mice, 8–12 weeks of age, weighing 20–25 g were obtained from the Animal Resources Centre (Perth, W.A., Australia) and used as both pancreatic islet donors and islet recipients. Mice were housed in an approved facility with a 12-hour day/night cycle with access to food and water ad libitum. All experiments carried out on mice were with prior approval of the Animal Ethics Committee, St. Vincent's Hospital, Melbourne, Vic., Australia, under the guidelines of the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm).

Materials

Gelitaspon (Gelita Medical BV, Amsterdam, The Netherlands) is a biodegradable sponge made from purified protein obtained by the partial acid hydrolysis of porcine collagen. All other chemicals were purchased from Sigma Aldrich Pty. (Sydney, N.S.W., Australia) unless otherwise stated.

Induction of Diabetes

Diabetes was induced in C57BL/6J mice by a single intraperitoneal injection of STZ (250 mg/kg body weight). STZ-induced diabetic mice are widely used as an animal model for diabetes, and have been fully characterised [Hayashi et al., 2006]. STZ is specifically taken up by β cells promoting cell death by necrosis. A single bolus dose induces fulminate diabetes in susceptible strains producing a disease that has resemblance in many aspects to non-obese diabetic mice and human insulin-dependent diabetes mellitus. Diabetes in C57BL/6J mice was confirmed by non-fasting blood glucose (NFBG) readings taken from the tail vein using a glucometre (Medisense Optium Xceed; Abbott Laboratories, Melbourne, Vic., Australia) on 2 successive days of ≥18 mmol/l.

Diabetic mice with BG levels ≥18 mmol/l and with a weight of >18 g were randomly assigned to the following experimental groups: (1) Gelitaspon + NGF + islets (n = 5), (2) Gelitaspon + islets (n = 4) and (3) Gelitaspon only (n = 6).

Islet Isolation

C57BL/6J mice were euthanised by cervical dislocation and islets isolated by bile duct cannulation and collagenase digestion (Collagenase P; Roche Diagnostics, Mannheim, Germany) of the pancreas. Islets were purified by density gradient (Histopaque-1077; Sigma Aldrich Pty.) and resuspended in CMRL 1066 media (Life Technologies, Gaithersburg, Md., USA) supplemented with 10% foetal calf serum, 200 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, pH 7.4. The isolated islets were hand picked using a stereomicroscope (Nikon Eclipse 55i; Nikon Tec Corp., Tokyo, Japan), and cultured overnight in supplemented CMRL media at 37°C in humidified 5% CO_2/95% air.

The Surgical Model

All surgery on diabetic C57BL/6J mice was performed under aseptic conditions using 2% isoflurane inhalational anaesthesia with supplemental oxygen (2 l/min) and performed using an operating microscope (Carl Zeiss Inc., Jena, Germany). Implantation of the chambers was based on a modified method as described previously [Cronin et al., 2004]. Each experimental group was implanted with chambers made from silicone tubing (Dow-Corning Corp., Midland, Mich., USA) with an internal volume of 44 μl. Chambers were split along their length to enfold around the femoral vessels and nerve. This neurovascular bundle was mobilised from the underlying fascial layer by ligating side branches to site the chamber. Five hundred islets were seeded onto (1) Gelitaspon discs (fig. 1) soaked with phosphate buffered saline (PBS), pH 7.4 (PBS) containing 100 ng/ml NGF (BD Biosciences, Billerica, Mass., USA) (Gelitaspon + islets + NGF group; n = 5) and (2) Gelitaspon discs soaked in PBS (Gelitaspon + islets group; n = 4). A final group of diabetic mice had a Gelitaspon disc soaked in PBS, but with no islets or NGF (Gelitaspon only group; n = 6).

Gelitaspon only or Gelitaspon with islets in the absence or presence of NGF was transferred into the chamber (fig. 2) and the silicone tubing was then sealed along its length with bone wax (Ethicon Inc., Somerville, N.J., USA). The distal end was closed by mobilising the inguinal fat pad and incorporating it into the distal end of the chamber. The proximal end was sealed with bone wax and the wound closed with metal clips.

Monitoring the Diabetic Mice

Mice were assessed weekly for NFBG as described above throughout the treatment period. At 8 weeks after transplantation all mice underwent an intraperitoneal glucose tolerance test (IPGTT). Mice were fasted and cage bedding material removed (but water allowed) for 18 h prior to the test. Fasting BG levels were determined at commencement to provide a baseline reading. D-glucose (300 mg/ml in saline) was injected intraperitoneally at a dose of 2 mg/g body weight. BG was measured at 15, 30, 45, 60, 120 and 180 min. For comparison, a cohort of non-diabetic C57BL/6J mice (n = 6) also underwent an IPGTT.

Harvesting the Graft Tissue

On completion of the IPGTT, mice were anaesthetised using 2% isoflurane inhalational anaesthesia with supplemental oxygen (2 l/min). The chambers were harvested surgically by ligating the femoral vessels, and pancreata harvested for further histological analysis. After removal of the chamber and pancreata, mice were then euthanised with an intracardiac injection of 0.3 ml pentobarbitone (Lethobarb; Virbac, Milperra, N.S.W., Australia).

Histological and Immunohistochemical Analysis

All tissue was fixed in 4% paraformaldehyde at 4°C overnight before being processed and paraffin embedded. Serial 5-μm sections were mounted onto slides coated with 3-aminopropyltriethoxysilane and dried overnight at 37°C.

Serial chamber graft and pancreatic sections were deparaffinised and rehydrated. Sections for routine histological morphology were visualized by staining with Mayer's haemotoxylin and eosin (HE) and with Giemsa solution for lymphocyte staining. Sections were mounted with a glass cover slip and Depex.

Insulin was detected on every 100-μm section using a DAKO Autostainer (Dako Cytomation, Carpenteria, Calif., USA). Sec-

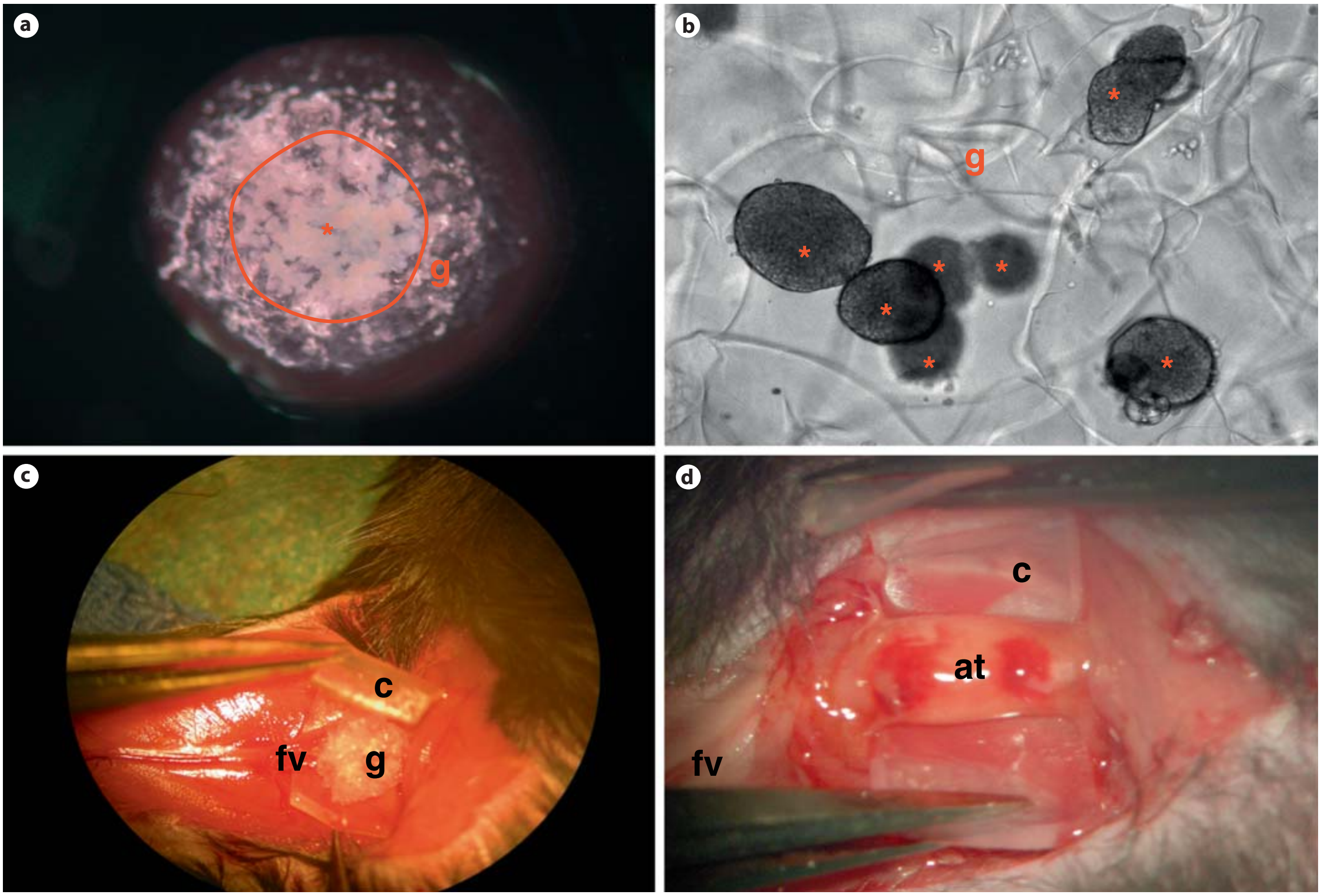


Fig. 1. a, **b** Phase contrast images of NGF supplemented Gelitaspon seeded with islets prior to transplantation within the chamber at ×4 objective (**a**) and the same image at ×20 objective (**b**). * = Islet; g = Gelitaspon. Red line encircles the position of islets within the Gelitaspon. **c**, **d** Islet grafts during surgery and at 12 weeks after transplantation in an STZ-induced diabetic mouse. **c** Islets seeded onto NGF-supplemented Gelitaspon in an open silicone chamber enclosing the femoral vessels and nerve prior to sealing the chamber with bone wax. **d** Islet graft at 12 weeks after chamber transplantation in an STZ-induced diabetic mouse showing well-vascularised adipose tissue. Photographs were taken using an operating microscope (Carl Zeiss). fv = Femoral vessels; g = NGF supplemented Gelitaspon with islets; c = silicone chamber; at = vascularised adipose tissue.

tions were blocked for endogenous peroxidase with 3% hydrogen peroxide in 50% methanol, followed by antigen retrieval using proteinase K (Dako Cytomation). Sections were incubated in a polyclonal antibody directed against guinea pig insulin (1:100 dilution; Dako Cytomation) for 1 h. Secondary antibody labeling was achieved by using rabbit immunoglobulin conjugated to peroxidase-labelled polymer (Rabbit Envision; Dako Cytomation) for 30 min and reactivity was visualized using diaminobenzaldehyde (DAB).

For detection of macrophages, sections were blocked for endogenous peroxidase with 3% hydrogen peroxide and blocked for non-specific binding before adding a monoclonal antibody directed against mouse F4/80 (1:500 dilution; Abcam Inc., Cambridge, Mass., USA) diluted in 5% normal rabbit serum for 1 h. Secondary detection was achieved through incubation with a rabbit antibody directed against rat biotin (1:200; Dako Cytomation) followed by HRP-streptavidin (1:400). Reactivity was visualized using DAB.

Detection of sympathetic nerves was achieved by heat-induced antigen retrieval by boiling in 10 mM citrate buffer, pH 6.0. Sections were blocked for endogenous peroxidase with 3% hydrogen peroxide and non-specific binding followed by a rabbit polyclonal antibody directed against rat tyrosine hydroxylase (1:500 dilution) for 1 h. Secondary detection was achieved through incubation with a goat biotinylated antibody directed against rabbit IgG (1:200 dilution; Vector Laboratories Inc., Burlingame, Calif., USA) followed by HRP-streptavidin (1:400). Reactivity was visualised with DAB.

All sections were counterstained with Mayer's haematoxylin and mounted with a glass cover slip and Depex.

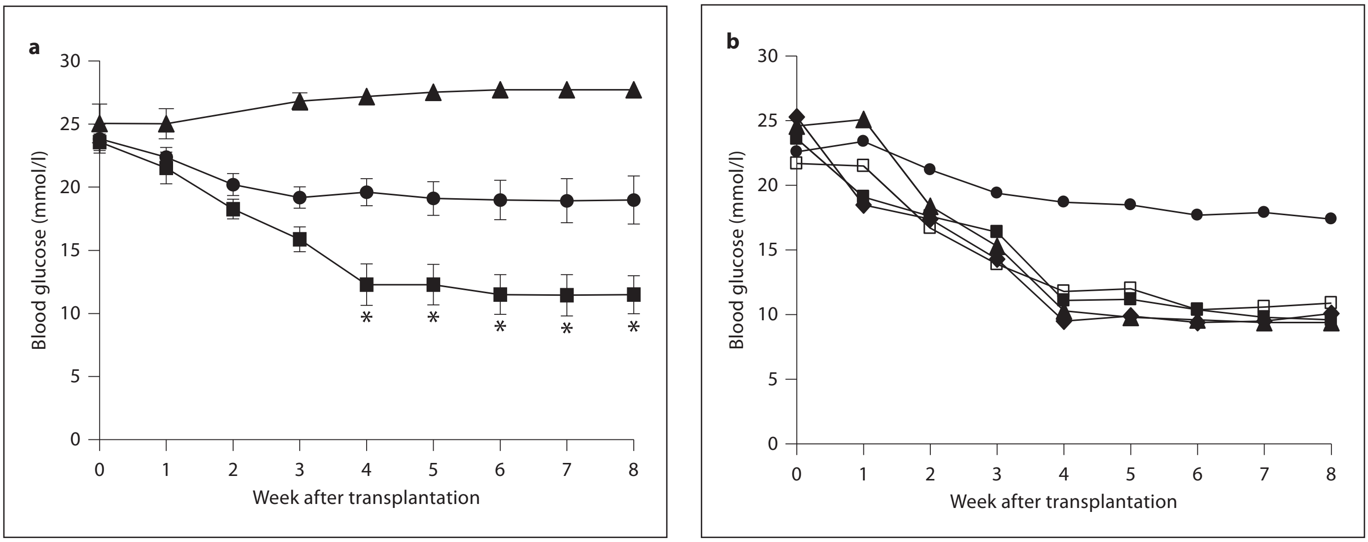


Fig. 2. a Weekly BG measurements from STZ-induced diabetic mice with chambers containing Gelitaspon only (triangle; n = 6), with chambers containing Gelitaspon + islets (circle; n = 4) and with chambers containing Gelitaspon + NGF + islets (square; n = 5) over 8 weeks after transplantation. Diabetic mice transplanted with chambers containing Gelitaspon + NGF + islets show a significant reduction in BG levels compared to diabetic mice transplanted with chambers containing Gelitaspon + islets and diabetic mice transplanted with chambers containing Gelitaspon only ($p < 0.001$, repeated measures two-way ANOVA) and the direct comparison of Gelitaspon + islets and Gelitaspon + NGF + islets is also significant ($p = 0.005$). Data points are mean ± SEM. * $p < 0.05$ by t test; **b** Weekly BG measurements for each STZ-induced diabetic mice with chambers containing Gelitaspon + NGF + islets over 8 weeks after transplantation (n = 5). Four of five diabetic mice show a return close to normoglycaemic levels.

TUNEL Analysis

Detection of apoptosis was performed using a DeadEnd™ Colorimetric TUNEL System (Promega Corporation, Madison, Wisc., USA). Sections from chamber islet grafts were deparaffinised, rehydrated and antigen retrieval achieved using proteinase K. Sections were equilibrated with buffer before adding recombinant terminal deoxynucleotidyl transferase reaction mix for 1 h at 37°C. Reaction was terminated by immersing slides in sodium citrate solution. Slides were washed in PBS and sections blocked for endogenous peroxidase. Detection of TUNEL-positive cells was achieved through an avidin-biotin-HRP detection system (ABC Elite; Vector Laboratories Inc.) and reactivity visualized using DAB. All sections were counterstained with Mayer's haematoxylin and mounted with a glass cover slip and Depex.

Statistics

Results are reported as mean ± SEM for the total number of observations, where n equals the number of mice used. Results were assessed by two-way mixed factorial analysis of variance (ANOVA) with the glucose measurements in the same animal in weekly BG or IPGTT treated as repeated measures and the treatment groups as independent measures. A Dunnett t test was used post hoc where there was a single obvious control group or Student-Newman-Keuls' test where there was not. Values of $p < 0.05$ were considered as statistically significant.

Results

All mice in both the experimental and control groups survived. In the chamber groups the femoral vascular pedicle was found to be patent in all animals at the time of harvest.

NGF Restores Glucose Levels in STZ-Induced Diabetic Mice

The minimal number of islets transplanted beneath the renal capsule that resulted in a return to normoglycaemic levels in STZ-induced diabetic mice was 500 [unpubl. data]. Diabetic mice transplanted with Gelitaspon only or with 500 islets on Gelitaspon into a flow through tissue engineering chamber, sited on the femoral vessels remained hyperglycaemic for the duration of the study. In contrast, mice that were transplanted with 500 islets on Gelitaspon in the presence of 100 ng/ml NGF demonstrated a gradual decrease in BG (fig. 2a). Four of five mice showed a reduction in hyperglycaemic levels from >20 mmol/l to <11.9 mmol/l by 4 weeks after transplantation (fig. 2b) with a mean level of 12.28 ± 1.65 mmol/l (n = 5) compared with the Gelitaspon with islets group

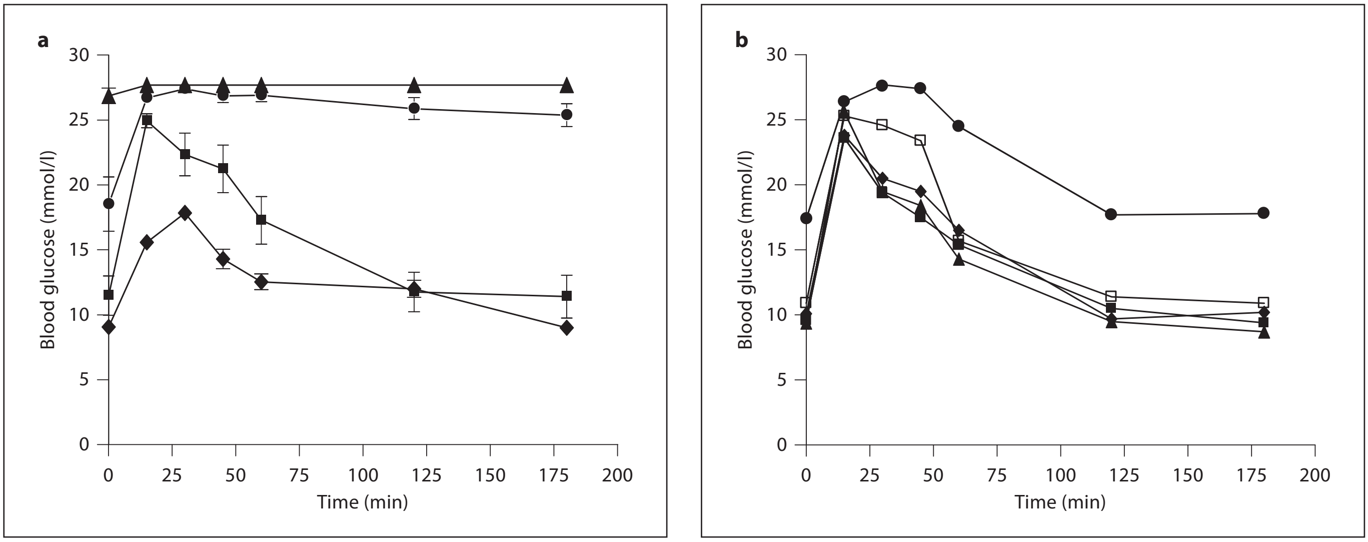


Fig. 3. a IPGTT results for STZ-induced diabetic mice with chambers containing Gelitaspon only (triangle; n = 6), with chambers containing Gelitaspon + islets (circle; n = 4), with chambers containing Gelitaspon + NGF + islets (square; n = 5) and non-diabetic control mice (diamond; n = 5). The Gelitaspon + NGF + islet group shows an improved response compared to Gelitaspon + islets ($p < 0.05$, two-way repeated measures ANOVA, post hoc SNK) and although the Gelitaspon + NGF + islet group had a similar response to that of non-diabetic controls there was still a statistically significant difference ($p < 0.05$). Data points are mean ± SEM; **b** IPGTT results for all STZ-induced diabetic mice with chambers containing Gelitaspon + NGF + islets (n = 5).

which was 20.9 ± 1.33 mmol/l (n = 4). By 7 weeks after transplantation, BG of the Gelitaspon + islet + NGF group had further decreased to a mean of 11.50 ± 1.56 mmo/l (n = 5) and remained at this level for the remainder of the treatment period (fig. 2a). The difference in weekly BG between the two groups was statistically significant (p = 0.005).

STZ-Induced Diabetic Mice Transplanted with Islets and NGF Show a Relatively Normal Response to an IPGTT

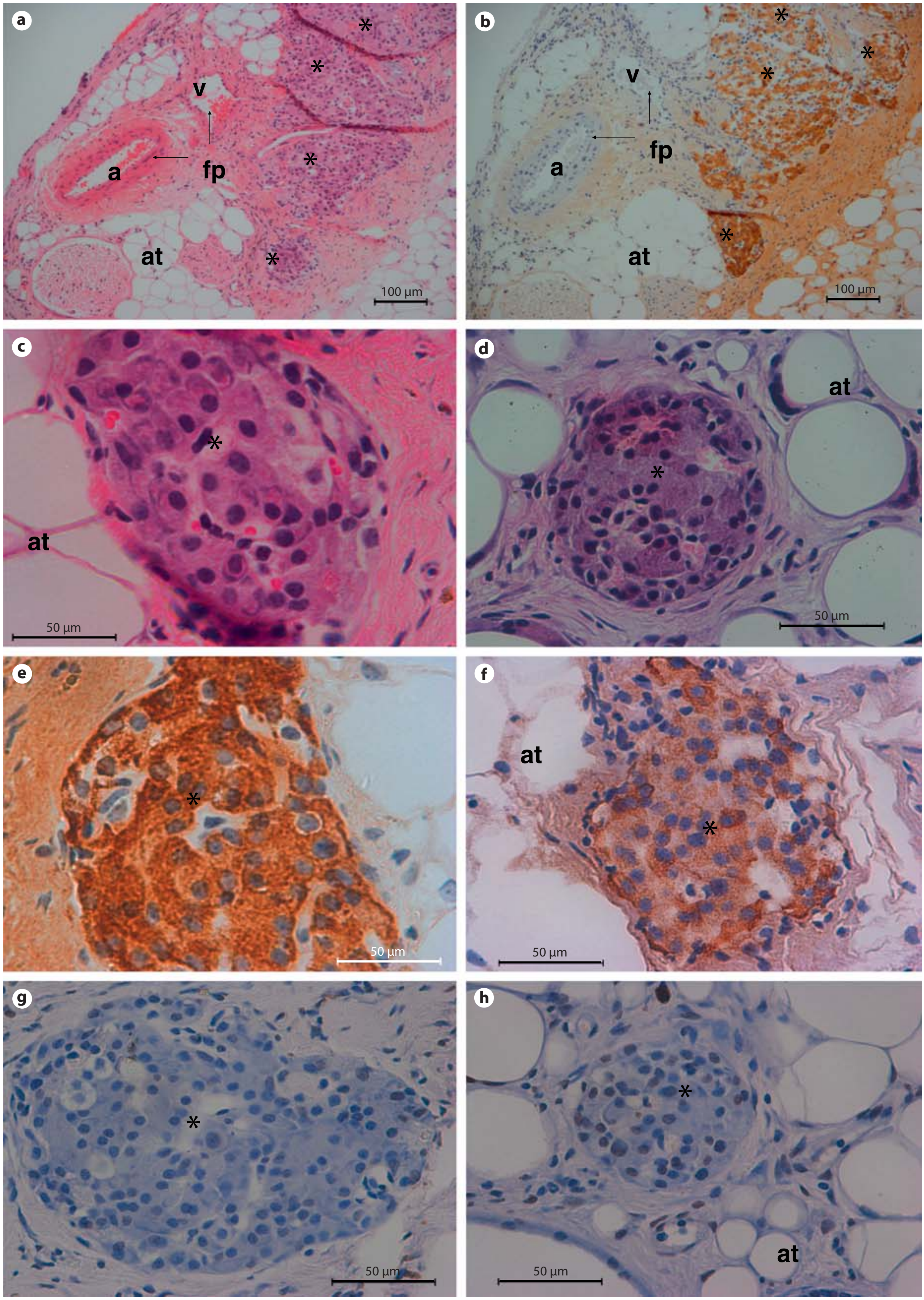
To further evaluate the function of transplanted islets an IPGTT was performed on all groups of mice at 8 weeks after transplantation, and on age-matched non-diabetic controls (fig. 3a). Four of five mice transplanted with islets on Gelitaspon in the presence of NGF demonstrated a response similar to non-diabetic controls, initially rising higher but returning to baseline levels at completion of the IPGTT (fig. 3b). This group of mice was significantly different when compared to mice transplanted with islets on Gelitaspon or Gelitaspon only groups. Both of these later groups remained hyperglycaemic for the duration of the IPGTT, demonstrating a diminished capacity for glucose tolerance (fig. 3a).

Islet Survival Is Enhanced with NGF

Haematoxylin and eosin staining of serial sections of the Gelitaspon with islets and NGF containing chamber grafts revealed multiple islets at 8 weeks after transplantation (fig. 4a, c). These islets generally retained an intact rounded appearance or aggregates of multiple islets with a disrupted architecture located adjacent to the femoral pedicle, and surrounded by mature vascularised adipocytes (fig. 4a). Immunohistochemical staining for insulin demonstrated abundant β cells within these islets (fig. 4b, e). In comparison, there were few islets detected in sections from chambers obtained from the Gelitaspon + islet group, and these islets appeared to show less intense staining for insulin, suggesting a compromised function (fig. 4f). Cells within islets from both groups were negative for apoptosis (fig. 4g, h).

Immunohistochemical staining for insulin production in serial sections of pancreata harvested from the chamber groups was negative, demonstrating that STZ specifically lead to death of β cells in the pancreas. This supports the finding that the improved glycaemic and glucose tolerance was due to the islet grafts.

Staining for F4/80 showed intense staining for macrophages within the fibrous capsule surrounding the grafts of both groups (fig. 5a, e). Macrophages were observed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surrounding some islets within the grafts from the Gelitaspon + islet group, but not within grafts from the Gelitaspon + islet + NGF group (fig. 5b, f). Lymphocytes were observed within grafts from both the islet groups in close proximity to blood vessels, but not surrounding islets (fig. 5c, d, g, h).

NGF Improves Nerve Reinnervation of Transplanted Islets

To evaluate reinnervation within transplanted islets, sections from islet grafts were stained for tyrosine hydroxylase (TH). Nerve fibres positive for TH were observed distributed within islets treated with NGF (fig. 6c, d). Nerve fibres positive for TH were also observed within untreated islets, located around the periphery of the islet (fig. 6b).

Discussion

This study has shown that the biodegradable scaffold Gelitaspon, combined with NGF and placed into a subcutaneously sited vascularised tissue-engineering chamber around the femoral neurovascular pedicle provides a suitable vehicle for islet transplantation in a murine model of diabetes. We demonstrate that diabetic mice transplanted with islets seeded onto Gelitaspon with NGF show a significant reduction in weekly NFBG, and when challenged with an IPGTT show a response close to that of non-diabetic mice. This subcutaneous, accessible and protected chamber technique of islet delivery has several attractions. Gelitaspon is an absorbable gelatin sponge of porcine origin approved by the FDA, and is currently used clinically in surgical wounds. α-Gelatin, the major component, is a purified protein obtained by the partial acidic hydrolysis of collagen, and contains an amino acid composition homologous to human collagen and is readily biodegradable. It has the advantage of being relatively cheap, easily manufactured and is suitable as a carrier for large quantities of cells allowing in-growth of vessels and diffusion of oxygen and nutrients from recipient tissues.

Other macroporous gelatin scaffolds have been utilised as islet carriers successfully in vitro and in vivo. Dispersed bovine islets cultured in gelatin beads maintained their capacity for increased insulin secretion after a glucose challenge [Del Guerra et al., 2001]. In a recent study, diabetic mice transplanted with islets, seeded into collagen IV-coated macroporous gelatin scaffolds into the epididymal fat pad achieved euglycaemia that was maintained for up to 297 days, and showed a normal glucose response when challenged with an IPGTT [Salvay et al., 2008]. Encouraging results have also been shown in clinical trials with the transplantation of autologous keratinocytes seeded into gelatin microbeads in the healing of chronic leg ulcers [Liu et al., 2004]. Our study using Gelitaspon as the scaffold demonstrated islet survival and function in the short term. The effects of long-term studies on islet survival and function are still to be undertaken.

Fig. 4. Histological and immunohistochemical staining for HE, insulin and TUNEL in grafts from STZ-induced diabetic mice transplanted with chambers containing Gelitaspon + NGF + islets or Gelitaspon + islets. Grafts from the Gelitaspon + NGF + islets group stained for HE (**a**) and insulin (**b**). Islets are located close to the femoral pedicle and stain intensely for insulin; ×10 objective; bar in micrographs represents 100 μm. Graft from the Gelitaspon + NGF + islet group stained for HE (**c**), insulin (**e**) and TUNEL (**g**). Islet shows intense staining for insulin and is negative for TUNEL; ×40 objective. Graft from the Gelitaspon + islet group stained for HE (**d**), insulin (**f**) and TUNEL (**h**). Islet shows a reduced staining for insulin and is negative for TUNEL; ×40 objectives. at = Adipose tissue; * = islets; fp = femoral pedicle; fn = femoral nerve; a = artery; v = vein. Bars in micrographs represents 50 μm. Micrographs are representative of 3 separate serial sections.

Fig. 5. Histological and Immunohistochemical staining for inflammatory cells in grafts from STZ-induced diabetic mice transplanted with chambers containing Gelitaspon + NGF + islet or Gelitaspon + islet. **a–d** Grafts from the Gelitaspon + NGF + islet group stained for macrophages with anti-F4/80; ×40 objective (**a**, **b**); lymphocytes using a Giemsa stain; ×40 objective (**c**, **d**). Grafts showed intense staining for macrophages within the fibrous capsule surrounding the graft and lymphocytes were observed within the graft close to vessels. Macrophages and lymphocytes were not observed near islets. Grafts from the Gelitaspon + islet group stained for macrophages with anti-F4/80; ×40 objective (**e**, **f**); lymphocytes using a Giemsa stain; ×40 objective (**g**, **h**). Grafts showed intense staining for macrophages within the fibrous capsule surrounding the grafts and surrounding some islets within the grafts and lymphocytes were observed within the graft close to vessels. Lymphocytes were not observed near islets. Arrows show macrophages. * = Islets; l = lymphocytes; v = vessel. Bars in micrographs represent 50 μm. Micrographs are representative of 3 serial sections.
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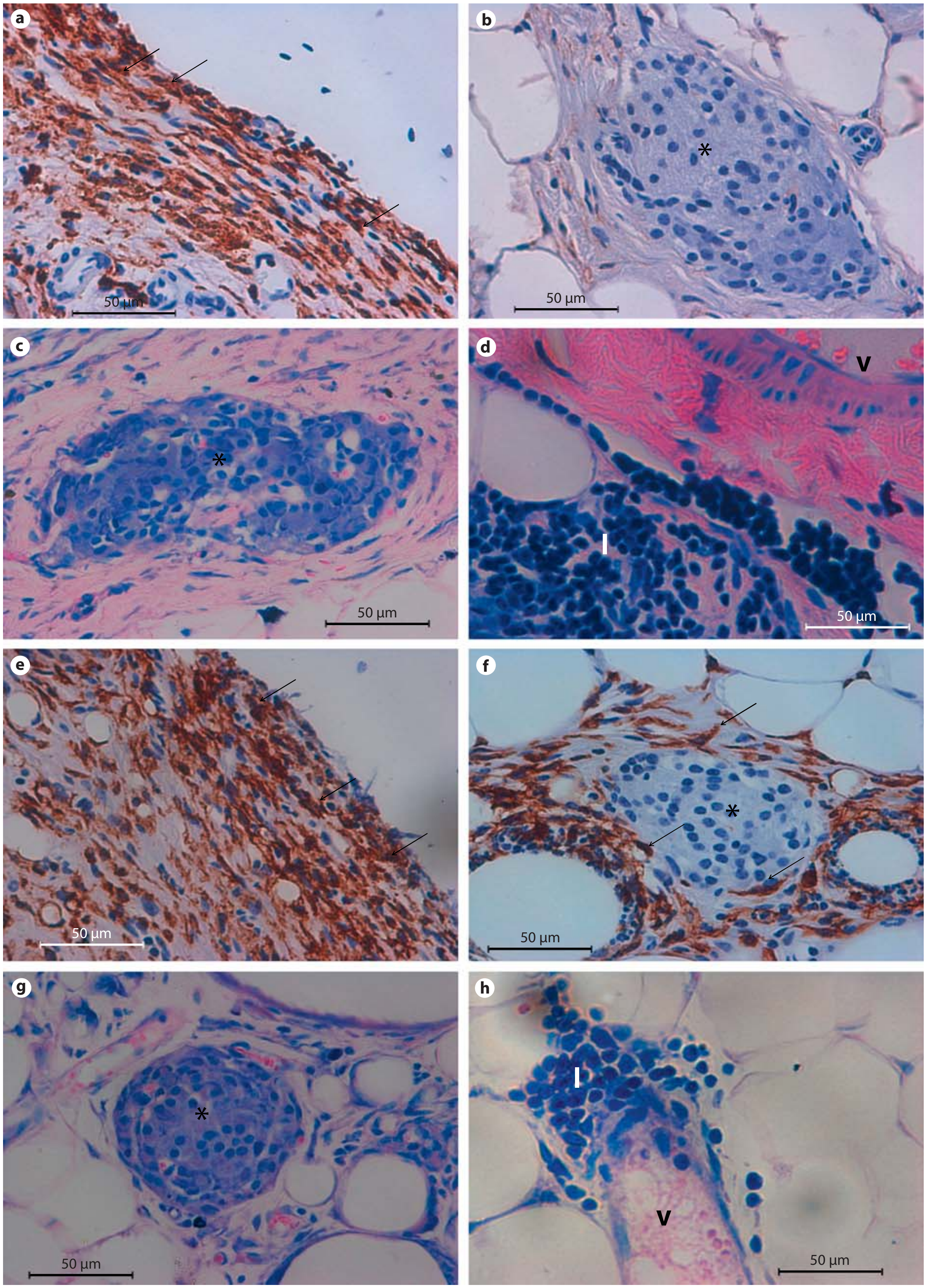

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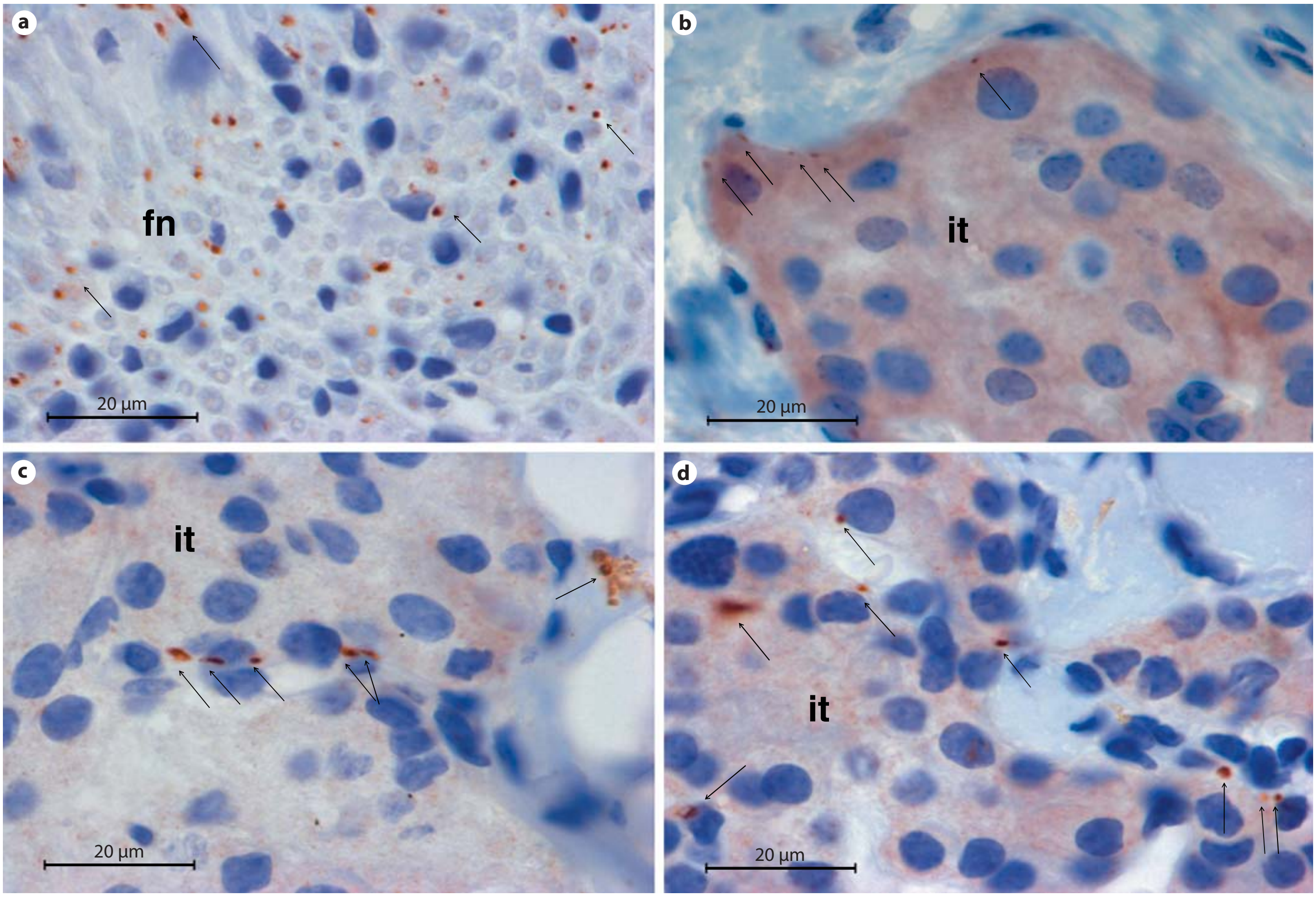

Fig. 6. Immunohistochemical staining for nerves using an antibody directed against TH in grafts from STZ-induced diabetic mice transplanted with Gelitaspon + islets in the presence or absence of NGF; **a** femoral nerve showing fibers positive for TH (arrows); **b** graft from the Gelitaspon + islet group showing 5 nerves around the periphery of the islet tissue (arrows); **c**, **d** grafts from the Gelitaspon + islet + NGF group showing nerves in the islet tissue (arrows).

Subcutaneous sites are attractive for islet transplantation as they require less invasive procedures. Successful subcutaneous islet transplantation has, however, been difficult to achieve requiring either a period of pre-vascularisation before transplantation [Pileggi et al., 2006], or islet encapsulation strategies [Kawakami et al., 2000] to treat diabetic rodents. For example, in a recent study by Pileggi et al. [2006], a biocompatible, cylindrical stainless steel mesh device was used to create a well-vascularised environment prior to islet transplantation in diabetic rats. The diabetic rats demonstrated a return to normoglycaemic levels that was maintained for more than 160 days. Islet transplantation under the kidney capsule [Morini et al., 2007], epididymal fat pad [Chen et al., 2007] and spleen [Gustavson et al., 2005] have been successful in reversing diabetes in rodents. However, these sites are not ideal in the clinical setting. Vascularisation is the key to islet survival and the design of the chamber used in this study focuses on this. The islet seeded NGF and Gelitaspon discs are brought into close proximity immediately adjacent to a rapidly expanding rich blood supply so that a pre-vascularisation period is not required. This is supported by a recent clinical study by Prochorov et al. [2008], in which transplantation of encapsulated rabbit islets (>6,000 IEQs) into a vascular lumen reduced insulin dependence in 73.7% of the patients within 2 years.

NGF is one of many intrinsic factors that support pancreatic islet function and survival. As well as having neurotrophic actions, NGF plays an important regulatory role in β cell function. NGF has been shown to modulate β cell plasticity by promoting neurite-like outgrowth in primary cultures of foetal and adult β cells [Vidaltamayo et al., 1996] and in insulinoma cell lines [Polak et al., 1993], and has been shown to enhance glucose-stimulated insulin secretion from the INS-1 insulinoma cell line [Frodin et al., 1995]. Cultured human β cells express NGF as well as the NGF receptors gp140$^{Trk\text{-}A}$ and p75^{NTR} [Pierucci et al., 2001]. Blockage of these receptors in β cells with an anti-NGF antibody or NGF withdrawal induces apoptosis in vitro. In recent studies, islets cultured with up to 500 ng/ml NGF showed improved viability, by inhibiting apoptosis, and improved insulin secretory function. NGF-treated islets transplanted beneath the renal capsule of diabetic mice showed a significantly improved glucose tolerance compared to non-NGF-treated islets [Miao et al., 2005, 2006]. Furthermore, local NGF treatment of islets was more effective at reversing diabetes in mice than by systemic application [Miao et al. 2006].

NGF has also been shown to induce a potent angiogenic response in vitro [Cantarella et al., 2002] and promote angiogenesis and arteriogenesis in ischaemic hind limbs accelerating tissue recovery, when delivered daily over 14 days after ischaemia [Emanueli et al., 2002]. We quantified percent vascular volume within islets in our study (data not shown), and found no statistically significant differences at 12 weeks after transplantation between grafts in the presence or absence of NGF.

NGF, delivered by controlled release, has been shown to enhance sciatic nerve regeneration [Lee et al., 2003]. The observed beneficial effect of NGF on islet function in our chamber model could be due to an NGF-induced increase in autonomic nerve growth into the transplanted islets. Sections from our chamber grafts stained for the sympathetic marker TH did show some difference in expression within islets through the addition of NGF. However, these results remain inconclusive as better islet survival may itself have led to better innervation. Further histological investigations including staining for vesicular monoamine transporter will possibly allow for a more quantitative assay of sympathetic islet innervation.

In our study, diabetic mice transplanted with islets seeded onto Gelitaspon in the presence of NGF showed a significant reduction in NFBG and a response to an IPGTT close to that seen in non-diabetic controls, compared with islets in the absence of NGF, but did observe intense staining for insulin and difference in TH staining of nerves within islets treated with NGF compared with non-treated islets. Given our mode of NGF delivery, one bolus dose at time of transplantation with Gelitaspon and islets, we conclude that in our study NGF may have improved islet viability and secretory function in our tissue-engineering chamber model. This chamber model has the advantage that if previous islet transplantation attempts have resulted in poor islet engraftment, there is an opportunity to transplant more islets by accessing the existing wound. Alternatively, if there are any adverse reactions experienced as a result of this method, the chamber can be easily removed. In conclusion, we have demonstrated in this study that islets are efficiently transplanted in vascularised chambers based on the neurovascular femoral artery, vein and nerve, in the presence of a gelatin sponge scaffold with NGF. Syngeneic islets transplanted with this method were capable of successfully maintaining glycaemic control in STZ-induced diabetic mice in short-term studies. For future investigation, we intend to conduct long-term studies using Gelitaspon and NGF, and to examine how allogeneic islets can also be immune protected using this model of islet transplantation.

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